PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark
Office, PCT
2011 South Clark Place Room
CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 26 June 2001 (26.06.01)	
International application No. PCT/EP00/07696	**Applicant's or agent's file reference** NO 6594/WO
International filing date (day/month/year) 08 August 2000 (08.08.00)	**Priority date** (day/month/year) 27 August 1999 (27.08.99)
Applicant BRUESSOW, Harald et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   22 March 2001 (22.03.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

Christine Carrié

Telephone No.: (41-22) 338.83.38

Form PCT/IB/331 (July 1992)

Copy for the Elected Office (EO/US)

PCT/EP00/07696

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF THE RECORDING OF A CHANGE

(PCT Rule 92bis.1 and Administrative Instructions, Section 422)

To:

STRAUS, Alexander
Becker-Kurig-Straus
Bavariastrasse 7
D-80336 Munich
ALLEMAGNE

Date of mailing (day/month/year)
24 August 2001 (24.08.01)

Applicant's or agent's file reference
NO 6594/WO

IMPORTANT NOTIFICATION

International application No.
PCT/EP00/07696

International filing date (day/month/year)
08 August 2000 (08.08.00)

1. The following indications appeared on record concerning:

[ ] the applicant [ ] the inventor [X] the agent [ ] the common representative

Name and Address
LOCK, Graham
55, Avenue Nestlé
CH-1800 Vevey
Switzerland

State of Nationality:
State of Residence:
Telephone No.: +41 21 924 47 60
Facsimile No.: +41 21 924 28 80
Teleprinter No.:

2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning:

[X] the person [X] the name [X] the address [ ] the nationality [ ] the residence

Name and Address
STRAUS, Alexander
Becker-Kurig-Straus
Bavariastrasse 7
D-80336 Munich
Germany

State of Nationality:
State of Residence:
Telephone No.: (089) 746303-0
Facsimile No.: (089) 746303-11
Teleprinter No.:

3. Further observations, if necessary:

4. A copy of this notification has been sent to:

[X] the receiving Office
[ ] the International Searching Authority
[X] the International Preliminary Examining Authority
[ ] the designated Offices concerned
[X] the elected Offices concerned
[ ] other:

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer
Idhir BRITEL

Telephone No.: (41-22) 338.83.38

Form PCT/IB/306 (March 1994)

004234691

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
NO 6594/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/ 07696	08/08/2000	27/08/1999
Applicant		
SOCIETE DES PRODUITS NESTLE S.A.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

PHAGE RESISTANT STREPTOCOCCUS THERMOPHILUS

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 4

[X] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/07696

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N1/21 A23C9/12 A23C19/032 //(C12N1/21, C12R1:46)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STURINO J M ET AL: "Construction of bacteriphage resistant strains of Streptococcus thermophilus by pGh9::ISS1 insertional mutagenesis." JOURNAL OF DAIRY SCIENCE, vol. 81, no. SUPPL. 1, 1998, page 7 XP002163735 Joint Meeting of the American Dairy Science Association and the American Society of Animal Science;Denver, Colorado, USA; July 28-31, 1998 ISSN: 0022-0302 the whole document ---	1,3,4, 8-10
X	EP 0 748 871 A (NESTLE SA) 18 December 1996 (1996-12-18) claims 1-9; examples 1-4 ---	1-3,6, 8-10
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2001	09/04/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

1

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT [redacted] 00/07696

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	FOLEY SOPHEY ET AL: "A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to Streptococcus thermophilus." VIROLOGY, vol. 250, no. 2, 25 October 1998 (1998-10-25), pages 377-387, XP002163736 ISSN: 0042-6822 page 379 -page 380; figure 1; tables 1-3 ---	1-3,6,8
A	EP 0 183 469 A (GENENTECH INC) 4 June 1986 (1986-06-04) ---	
A	MAGUIN E ET AL: "EFFICIENT INSERTIONAL MUTAGENESIS IN LACTOCOCCI AND OTHER GRAM-POSITIVE BACTERIA" JOURNAL OF BACTERIOLOGY,US,WASHINGTON, DC, vol. 178, no. 3, 1 February 1996 (1996-02-01), pages 931-935, XP000673892 ISSN: 0021-9193 cited in the application ---	
T	LUCCHINI SACHA ET AL: "Broad-range bacteriophage resistance in Streptococcus thermophilus by insertional mutagenesis." VIROLOGY, vol. 275, no. 2, 30 September 2000 (2000-09-30), pages 267-277, XP002163737 ISSN: 0042-6822 -----	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT 00/07696

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0748871 A	18-12-1996	CA 2178975 A	17-12-1996
		JP 9000274 A	07-01-1997
		US 5766904 A	16-06-1998
EP 0183469 A	04-06-1986	JP 61132179 A	19-06-1986

PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
NO 6594/WO		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/EP 00/07696	08/08/2000	27/08/1999
Applicant		
SOCIETE DES PRODUITS NESTLE S.A.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

[ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

[ ] contained in the international application in written form.

[ ] filed together with the international application in computer readable form.

[X] furnished subsequently to this Authority in written form.

[X] furnished subsequently to this Authority in computer readble form.

[X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

[X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [X] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

[ ] the text is approved as submitted by the applicant.

[X] the text has been established by this Authority to read as follows:

PHAGE RESISTANT STREPTOCOCCUS THERMOPHILUS

5. With regard to the **abstract,**

[X] the text is approved as submitted by the applicant.

[ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. 4

[X] as suggested by the applicant.

[ ] because the applicant failed to suggest a figure.

[ ] because this figure better characterizes the invention.

[ ] None of the figures.

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N1/21 A23C9/12 A23C19/032 //(C12N1/21, C12R1:46)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STURINO J M ET AL: "Construction of bacteriphage resistant strains of Streptococcus thermophilus by pGh9::ISS1 insertional mutagenesis." JOURNAL OF DAIRY SCIENCE, vol. 81, no. SUPPL. 1, 1998, page 7 XP002163735 Joint Meeting of the American Dairy Science Association and the American Society of Animal Science;Denver, Colorado, USA; July 28-31, 1998 ISSN: 0022-0302 the whole document ---	1,3,4, 8-10
X	EP 0 748 871 A (NESTLE SA) 18 December 1996 (1996-12-18) claims 1-9; examples 1-4 --- -/--	1-3,6, 8-10

[X] Further documents are listed in the continuation of box C. [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 March 2001	09/04/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indic[redacted]here appropriate, of the relevant passages	Relevant to claim No.
X	FOLEY SOPHEY ET AL: "A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to Streptococcus thermophilus." VIROLOGY, vol. 250, no. 2, 25 October 1998 (1998-10-25), pages 377-387, XP002163736 ISSN: 0042-6822 page 379 -page 380; figure 1; tables 1-3 ---	1-3,6,8
A	EP 0 183 469 A (GENENTECH INC) 4 June 1986 (1986-06-04) ---	
A	MAGUIN E ET AL: "EFFICIENT INSERTIONAL MUTAGENESIS IN LACTOCOCCI AND OTHER GRAM-POSITIVE BACTERIA" JOURNAL OF BACTERIOLOGY,US,WASHINGTON, DC, vol. 178, no. 3, 1 February 1996 (1996-02-01), pages 931-935, XP000673892 ISSN: 0021-9193 cited in the application ---	
T	LUCCHINI SACHA ET AL: "Broad-range bacteriophage resistance in Streptococcus thermophilus by insertional mutagenesis." VIROLOGY, vol. 275, no. 2, 30 September 2000 (2000-09-30), pages 267-277, XP002163737 ISSN: 0042-6822 -----	

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: in part: 1,2,3,8,9,10; completely: 6,7

   S. thermophilus bacterium which is resistant to attack by at least one bacteriophage and which comprises a modification (a deletion) of the Sfi21 prophage; a method for preparing such a bacterium; a composition comprising such a bacterium.

2. Claims: in part: 1,2,3,8,9,10; completely: 4,5

   S. thermophilus bacterium which is resistant to attack by at least one bacteriophage and which comprises a modification (an ISS1 insertion) of the bacterial chromosome; a method for preparing such a bacterium; a composition comprising such a bacterium.

INTERNATIONAL SEARCH REPORT

PCT/EP 00/07696

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0748871 A	18-12-1996	CA 2178975 A	17-12-1996
		JP 9000274 A	07-01-1997
		US 5766904 A	16-06-1998
EP 0183469 A	04-06-1986	JP 61132179 A	19-06-1986

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PATENT COOPERATION TREATY

REC'D 12 NOV 2001

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
NO 6594/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/07696	08/08/2000	27/08/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/31		
Applicant		
SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/03/2001	08.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Trommsdorff, M Telephone No. +49 89 2399 7361

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/07696

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-16 as originally filed

Claims, No.:

1-10 as received on 31/08/2001 with letter of 31/08/2001

Drawings, sheets:

1/6-6/6 as originally filed

Sequence listing part of the description, pages:

1-2, filed with the letter of 13.11.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5, 7
	No:	Claims	1-4, 6, 8-10
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-10
Industrial applicability (IA)	Yes:	Claims	1-10
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. Cited documents

The following documents (D) are referred to in this communication; the numbering is the same as in the search report and will be adhered to in the rest of the procedure:

D1: Sturino J M et al: 'Construction of bacteriphage resistant strains of Streptococcus thermophilus by pGh9::ISS1 insertional mutagenesis.' JOURNAL OF DAIRY SCIENCE, vol. 81, no. SUPPL. 1, 1998, p.7 Joint Meeting of the American Dairy Science Association and the American Society of Animal Science; Denver, Colorado, USA; July 28-31, 1998 ISSN: 0022-0302

D2: EP-A-0 748 871 (NESTLE SA) 18 December 1996 (1996-12-18)

D3: Foley Sophey et al: 'A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to Streptococcus thermophilus.' VIROLOGY, vol.250, no.2, 25 October 1998 (1998-10-25), p.377-87 ISSN:0042-6822

2. Re Item I
Basis of the opinion

2.1. The amendments filed with the letter dated 31st August 200 do introduce subject-matter which extends beyond the content of the application as filed and thus do not comply with Article 34(2)(b) PCT for the following reasons:
the term "propagation" could not be found in the passages of the description cited as a basis for said amendment. The broad expression "such that phage propagation is prevented" embraces a series of possible mechanisms. The applicants however only show that for Sfi1-R24 mutant phage DNA replication is delayed and diminished (and thus directly or indirectly affected by chromosomal mutation) whereas for Sfi1-R7 and Sfi1-R71 mutants no phage replication is observed at all. The Sfi21 prophage mutants are inactive and thus do not even enter a replication phase.
Hence, since the only "propagation" event discussed is the replication step and since applicants only show that replication is diminished but not completely prevented, the expression "such that phage propagation is prevented" is too broad and goes beyond the scope of the description as filed.
Nevertheless, since other major objections remain, the amended claims were examined as filed and under the assumption that acceptable amendments will be filed in the event the present application enters the regional phase.

3. R. Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

3.1. The claims are directed to an *S thermophilus* bacterium mutated in the Sfi21 prophage or the bacterial chromosome, such that propagation within the bacterium is prevented.
D1 discloses the same method as in the application to generate bacteriophage resistant strains of *S thermophilus*, namely by pGh9::ISS1 insertional mutation. The obtained mutants are challenged with phage jud29, selected for their resistance to said phage and tested for ISS1 integration.
D1 shows that the main class of mutants absorb jud29 but that the efficiency of plaquing is strongly reduced. Thus, the phage enters the bacteria -at least in some cases- but its proliferation is prevented. D1 further states that indeed "the objective of this ongoing study is to characterize the native loci involved in lytic bacteriophage proliferation in *S thermophilus*".
Hence, the subject-matter of claims 1, 3, 4 and 8 lacks novelty over D1 (Art. 33(1) and (2) PCT).
Note that even if the phage was not absorbed and did not enter the bacteria, as a result of bacterial resistance, the phage would not be able to infect the bacteria and thus phage "propagation" would still be prevented.

3.2. D2 describes the generation of phage resistant *S thermophilus* strains by transformation of said bacteria with different genomic fragments of a ϕSfi21 phage. A Sfi1 strain that has been transformed with a 3.6kb HindIII fragment of the ϕSfi21 phage is shown to be resistant to said phage (p.6, table 1). Said resistant strain is used to inoculate a milk solution for the preparation of yoghurts (p.8, example IV). Here again, as a consequence of bacterial resistance to the phage, phage "propagation" is prevented.
Hence, D2 is prejudicial to the novelty of claims 1-3, 6, 8-10 (Art. 33(2) PCT).

3.3. D3 shows that a 302bp non-coding DNA fragment of ϕSfi21 (which can be considered as a deletion mutant of ϕSfi21) protects the *S thermophilus* strain Sfi1 from infection by 17 of 25 phages. The resistance seems to be copy number

dependent: the phage proteins interact with said inhibitory fragment and are thus no longer available for phage propagation (p.379, table 1, p.380, right column). In this document, it is clearly mentioned that phage propagation is prevented.
Thus, D3 is novelty destroying to the subject-matter of claims 1-3, 6 and 8 (Art. 33(2) PCT).

3.4. Claims 5 and 7 are directed to *S thermophilus* bacteria with disruptions in specific ORFs of the prophage.
Since these specific modifications are not disclosed in the prior art, said claims are novel (Art. 33(/1) and (2) PCT).
However, as discussed above, *S thermophilus* mutants bearing modifications of the prophage or bacterial genome which render them resistant to said phage and to homologue phages are taught in the prior art. Thus, the claimed mutants merely appear to be a further example of such resistant bacteria which do not show any further advantage over already known strains. Even if said mutants obtained by random mutagenesis are further characterized by the technical feature "that phage propagation within the baterium is prevented", this is not a novel feature over known mutants as discussed above. Furthermore, no technical advantage can be seen for said mutants compared to known mutants.
Hence, the subject-matter of claims 5 and 7 lacks an inventive step (Art. 33(1) and (3) PCT).

3.5. The subject-matter of claims 1-10 is industrially applicable in the field of food industry (Art. 33(4) PCT).

4. **Re Item VII**
Certain defects in the international application

4.1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D3 is not mentioned in the description, nor are these documents identified therein.

5. Re Item VIII
Certain observations on the international application

5.1. Claim 1 is too broad and not fully supported by the description for the following reasons (Art. 6 PCT):
the mutants claimed are not defined by any technical features. The claim rather attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result should be added. Otherwise it is impossible for the skilled person to identify which mutants fall into the scope of the claim. Since the method for generating said mutants is already known from the prior art and since not all mutants obtained by said method will solve the underlying problem, i.e. prevent phage propagation within the bacterium, the claim should be restricted to the specific mutants tested and which do solve the technical problem.

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

STRAUS, Alexander
BECKER-KURIG-STRAUS
Bavariastrasse 7
D-80336 München
ALLEMAGNE

BECKER KURIG STRAUS
BAVARIASTRASSE 7 · 80336 MÜNCHEN
- 9. Nov. 2001
WV: ..... / LF: .....

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	08.11.2001
Applicant's or agent's file reference NO 6594/WO 80308	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/07696	08/08/2000	27/08/1999

Applicant
SOCIETE DES PRODUITS NESTLE S.A. et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	MYLONAS, E Tel.+49 89 2399-7746

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
NO 6594/WO		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/EP00/07696	08/08/2000	27/08/1999
International Patent Classification (IPC) or national classification and IPC		
C12N15/31		
Applicant		
SOCIETE DES PRODUITS NESTLE S.A. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 1 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
22/03/2001	08.11.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Trommsdorff, M Telephone No. +49 89 2399 7361

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP00/07696

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, pages:

1-16	as originally filed				

Claims, No.:

1-10	as received on	31/08/2001	with letter of	31/08/2001	

Drawings, sheets:

1/6-6/6	as originally filed				

Sequence listing part of the description, pages:

1-2, filed with the letter of 13.11.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:
   see separate sheet

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	5, 7
	No:	Claims	1-4, 6, 8-10
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-10
Industrial applicability (IA)	Yes:	Claims	1-10
	No:	Claims	

2. Citations and explanations
   see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

1. Cited documents

The following documents (D) are referred to in this communication; the numbering is the same as in the search report and will be adhered to in the rest of the procedure:

D1: Sturino J M et al: 'Construction of bacteriphage resistant strains of Streptococcus thermophilus by pGh9::ISS1 insertional mutagenesis.' JOURNAL OF DAIRY SCIENCE, vol. 81, no. SUPPL. 1, 1998, p.7 Joint Meeting of the American Dairy Science Association and the American Society of Animal Science; Denver, Colorado, USA; July 28-31, 1998 ISSN: 0022-0302

D2: EP-A-0 748 871 (NESTLE SA) 18 December 1996 (1996-12-18)

D3: Foley Sophey et al: 'A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to Streptococcus thermophilus.' VIROLOGY, vol.250, no.2, 25 October 1998 (1998-10-25), p.377-87 ISSN:0042-6822

2. Re Item I
Basis of the opinion

2.1. The amendments filed with the letter dated 31st August 200 do introduce subject-matter which extends beyond the content of the application as filed and thus do not comply with Article 34(2)(b) PCT for the following reasons:
the term "propagation" could not be found in the passages of the description cited as a basis for said amendment. The broad expression "such that phage propagation is prevented" embraces a series of possible mechanisms. The applicants however only show that for Sfi1-R24 mutant phage DNA replication is delayed and diminished (and thus directly or indirectly affected by chromosomal mutation) whereas for Sfi1-R7 and Sfi1-R71 mutants no phage replication is observed at all. The Sfi21 prophage mutants are inactive and thus do not even enter a replication phase.
Hence, since the only "propagation" event discussed is the replication step and since applicants only show that replication is diminished but not completely prevented, the expression "such that phage propagation is prevented" is too broad and goes beyond the scope of the description as filed.
Nevertheless, since other major objections remain, the amended claims were examined as filed and under the assumption that acceptable amendments will be filed in the event the present application enters the regional phase.

3. R Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to nov lty, inventiv step or industrial applicability; citations and explanations supporting such statement

3.1. The claims are directed to an *S thermophilus* bacterium mutated in the Sfi21 prophage or the bacterial chromosome, such that propagation within the bacterium is prevented.
D1 discloses the same method as in the application to generate bacteriophage resistant strains of *S thermophilus*, namely by pGh9::ISS1 insertional mutation. The obtained mutants are challenged with phage jud29, selected for their resistance to said phage and tested for ISS1 integration.
D1 shows that the main class of mutants absorb jud29 but that the efficiency of plaquing is strongly reduced. Thus, the phage enters the bacteria -at least in some cases- but its proliferation is prevented. D1 further states that indeed "the objective of this ongoing study is to characterize the native loci involved in lytic bacteriophage proliferation in *S thermophilus*".
Hence, the subject-matter of claims 1, 3, 4 and 8 lacks novelty over D1 (Art. 33(1) and (2) PCT).
Note that even if the phage was not absorbed and did not enter the bacteria, as a result of bacterial resistance, the phage would not be able to infect the bacteria and thus phage "propagation" would still be prevented.

3.2. D2 describes the generation of phage resistant *S thermophilus* strains by transformation of said bacteria with different genomic fragments of a ϕSfi21 phage. A Sfi1 strain that has been transformed with a 3.6kb HindIII fragment of the ϕSfi21 phage is shown to be resistant to said phage (p.6, table 1). Said resistant strain is used to inoculate a milk solution for the preparation of yoghurts (p.8, example IV). Here again, as a consequence of bacterial resistance to the phage, phage "propagation" is prevented.
Hence, D2 is prejudicial to the novelty of claims 1-3, 6, 8-10 (Art. 33(2) PCT).

3.3. D3 shows that a 302bp non-coding DNA fragment of ϕSfi21 (which can be considered as a deletion mutant of ϕSfi21) protects the *S thermophilus* strain Sfi1 from infection by 17 of 25 phages. The resistance seems to be copy number

dependent: the phage proteins interact with said inhibitory fragment and are thus no longer available for phage propagation (p.379, table 1, p.380, right column). In this document, it is clearly mentioned that phage propagation is prevented. Thus, D3 is novelty destroying to the subject-matter of claims 1-3, 6 and 8 (Art. 33(2) PCT).

3.4. Claims 5 and 7 are directed to *S thermophilus* bacteria with disruptions in specific ORFs of the prophage.
Since these specific modifications are not disclosed in the prior art, said claims are novel (Art. 33(/1) and (2) PCT).
However, as discussed above, *S thermophilus* mutants bearing modifications of the prophage or bacterial genome which render them resistant to said phage and to homologue phages are taught in the prior art. Thus, the claimed mutants merely appear to be a further example of such resistant bacteria which do not show any further advantage over already known strains. Even if said mutants obtained by random mutagenesis are further characterized by the technical feature "that phage propagation within the baterium is prevented", this is not a novel feature over known mutants as discussed above. Furthermore, no technical advantage can be seen for said mutants compared to known mutants.
Hence, the subject-matter of claims 5 and 7 lacks an inventive step (Art. 33(1) and (3) PCT).

3.5. The subject-matter of claims 1-10 is industrially applicable in the field of food industry (Art. 33(4) PCT).

4. Re Item VII
Certain defects in the international application

4.1. Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1-D3 is not mentioned in the description, nor are these documents identified therein.

5. R Item VIII
Certain observations on th int rnational application

5.1. Claim 1 is too broad and not fully supported by the description for the following reasons (Art. 6 PCT):
the mutants claimed are not defined by any technical features. The claim rather attempts to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem. The technical features necessary for achieving this result should be added. Otherwise it is impossible for the skilled person to identify which mutants fall into the scope of the claim. Since the method for generating said mutants is already known from the prior art and since not all mutants obtained by said method will solve the underlying problem, i.e. prevent phage propagation within the bacterium, the claim should be restricted to the specific mutants tested and which do solve the technical problem.

Amended claims

1. A *S. thermophilus* bacterial strain, which has been mutated in the bacterial chromosome or in the φSfi21 prophage genome, such that phage propagation within the bacterium is prevented.

2. A bacterium according to claim 1 which is a strain of S. thermophilus selected from the group which consists of Sfi1 and Sfi1c16.

3. A bacterium according to claim 1 or claim 2 wherein the bacterial chromosome is modified by addition of DNA.

4. A bacterium according to claim 3 wherein the addition of DNA comprises the sequence of bases of ISS1 or a functional equivalent thereof.

5. A bacterium according to claim 3 or 4 wherein the bacterial chromosome comprises addition of DNA in ORF 90 at a site which disrupts expression of a chorismate mutase chain A gene and/or disrupts expression of the down stream gene ORF 394; or in ORF 269 at a site which disrupts expression of an oxidoreductase gene.

6. A bacterium according to claim 1 or 2 wherein the Sfi21 prophage is modified by deletion of sufficient DNA to disrupt expression of the prophage.

7. A bacterium according to claim 6 wherein the modification comprises a deletion of at least part of ORF 1560.

8. A method of preparing a bacterium according to any one of claims 1 to 7 which comprises the steps of disrupting expression of the bacterial chromosome or the Sfi21 prophage by inserting a DNA sequence or deleting a DNA sequence.

9. A composition which comprises the bacterium according to any one of claims 1 to 6 together with a carrier, adjuvant or diluent.

10. A composition according to claim 8 which is a starter culture or milk product.

Claims

1. An S thermophilus bacterium which is resistant to attack by at least one bacteriophage and which comprises a modification of the Sfi21 prophage or the bacterial chromosome.

2. A bacterium according to claim 1 which is a strain of S. thermophilus selected from the group which consists of Sfi1 and Sfi1c16.

3. A bacterium according to claim 1 or claim 2 wherein the bacterial chromosome is modified by addition of DNA.

4. A bacterium according to claim 3 wherein the addition of DNA comprises the sequence of bases of ISS1 or a functional equivalent thereof.

5. A bacterium according to claim 3 or 4 wherein the bacterial chromosome comprises addition of DNA in ORF 90 at a site which disrupts expression of a chorismate mutase chain A gene and/or disrupts expression of the down stream gene ORF 394; or in ORF 269 at a site which disrupts expression of an oxidoreductase gene.

6. A bacterium according to claim 1 or 2 wherein the Sfi21 prophage is modified by deletion of sufficient DNA to disrupt expression of the prophage.

7. A bacterium according to claim 6 wherein the modification comprises a deletion of at least part of ORF 1560.

8. A method of preparing a bacterium according to any one of claims 1 to 7 which comprises the steps of disrupting expression of the bacterial chromosome or the Sfi21 prophage by inserting a DNA sequence or deleting a DNA sequence.

9. A composition which comprises the bacterium according to any one of claims 1 to 6 together with a carrier, adjuvant or diluent.

10. A composition according to claim 8 which is a starter culture or milk product.

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(71) Applicant *(for all designated States except US)*: SOCIETE DES PRODUITS NESTLE S.A. [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BRUESSOW, Harald [DE/CH]; 13, Chemin de la Chaumény, CH-1814 La-Tour-de-Peilz (CH). LUCCHINI, Sacha [CH/GB]; 43, The Close, Norwich NR1 4EQ (GB).

(74) Agent: LOCK, Graham; 55, Avenue Nestlé, CH-1800 Vevey (CH).

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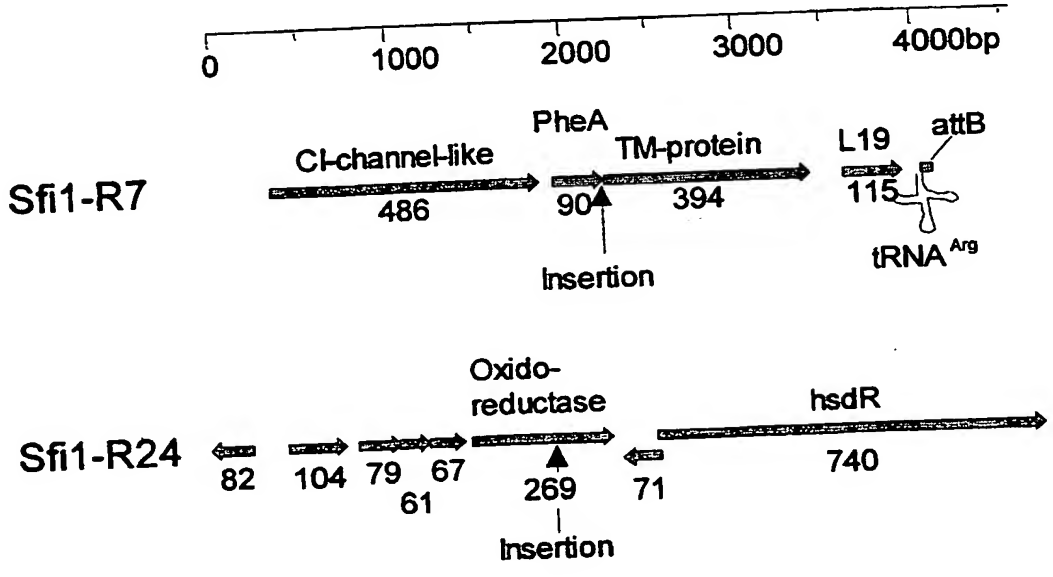
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: PHAGE RESISTANT BACTERIA



(57) Abstract: The invention relates to an S thermophilus bacterium which is restant to attack by at least one bacteriophage, a method of production of the bacterium and a composition which comprises the bacterium. The bacterium comprises a modification of the Sfi21 prophage or the bacterial genome which disrupts expression of a gene which is essential to a bacteriophage, but not essential to the bacteria.

WO 01/16329 A2

Phage Resistant Bacteria

The present invention relates to a bacterium which is resistant to attack by at least one bacteriophage, a method of production of the bacterium and a composition which comprises the bacterium.

Within the context of this specification the word "comprises" is taken to mean "includes, among other things". It is not intended to be construed as "consists of only".

DNA bases and amino acids are represented herein by their standard one or three letter abbreviations as defined by the IUPAC Biochemical Nomenclature Commission.

It is well known that bacteria are placed in starters for production of milk products. They are used to optimise pH and flavour of the milk product. Indeed, production of cheese and dairy products has long relied on the fermentation of milk by bacteria. The bacteria are responsible for the development of acid, flavour production, and often coagulum characteristics in mesophilic dairy fermentations. Because efficient milk fermentations are dependent on the growth and activity of the bacteria, great care is exercised to prepare starter cultures that are highly active and uncontaminated with undesirable microorganisms or bacteriophages. However, the fermentation process itself is nonaseptic, occurring in open vats with a nonsterile medium, pasteurized milk. It is therefore highly susceptible to contamination by bacteriophages. For the majority of strains employed in commercial dairy fermentations, bacteriophages capable of halting bacterial growth and acid production can appear within one to two days after introducing the culture.

Historically, milk fermentation has relied on starter cultures having mixtures of lactic acid bacteria. The presence of bacteriophages or protection from them was either unknown or missing. Natural phage contamination in these cultures is thought to have established an equilibrium of evolving bacteriophages and phage-resistant bacterial variants. The cultures were highly variable in levels of acid production, but remained moderately active and could be used in small fermentation factories. More recently, the dairy industry has become more aware

of starter culture failures due to bacteriophage infection and with an increasing demand for cultured milk products there is a need for an increase in both production capacity and process efficiency such that larger volumes of milk are processed and total processing time is shortened. This modernization of the industry has lead to an increased demand for uniform and rapid rates of acid production. However, the bacteria which may provide the best milk products may be highly susceptible to bacteriophage attack.

To cope with bacteriophage problems a number of methods have been developed to minimize phage infection in commercial milk fermentations. Through the use of concentrated cultures, aseptic bulk starter vessels and phage-inhibitory media (see, for example, U.S. Pat. No. 4,282,255), the starter culture can be protected from bacteriophage infection prior to vat inoculation. However, phage contamination cannot be prevented after introduction of the bacteria into the fermentation vessel.

Sandine, W. E., et al., J. Milk Food Technol. 35, 176 (1972) emphasized the need to isolate new bacterial strains for use in the dairy industry. Foremost among the criteria for selection of these strains was resistance to existing bacteriophages. It is now recognized that some strains of bacteria are not attacked by any phage when challenged with large collections of laboratory phage banks, or when used on a continuous, long-term basis in commercial fermentations. Some groups have reported the existence of bacteria that are not sensitive to bacteriophage attack. However, to date, only a limited number of phage-insensitive strains have been identified and studied for mechanism of phage resistance.

U.S. Pat. No. 4,530,904, discloses a method for protecting bacteria in general from different types of bacteriophage. The method involves transforming a bacterium with a recombinant DNA cloning vector which comprises a replicon that is functional in the bacterium, a gene that expresses a functional polypeptide (i.e., human growth hormone) in the bacterium, and a DNA segment which confers restriction and modification activity to the bacterium. The transformed bacterium is then cultured under large-scale fermentation conditions. This method is particularly adapted to fermentation procedures for the production of polypeptide products like growth hormone. Problems with this method are due to the fact that it relies on protection against phages provided by a restriction and

modification (R/M) phage resistance mechanism cloned on a plasmid. There are two main problems. First, a plasmid has high metabolic costs and also instability problems (insert can be deleted, plasmid can be lost in abscence of an appropriate selection). Second, restriction and modification systems (R/M) can be powerful; they operate at varying levels of efficiency (EOP of 10^{-1} - 10^{-6}). But, the problem with them is that they allow some phages to escape restriction resulting in modified progeny virions. The modified phages can then infect a second host bearing an identical R/M system without being restricted (EOP=1.0).

Phage contamination is now considered to be a main cause of slow fermentation or complete starter failure. The lack of bacteria which survive adequately can result in milk products which do not have a desirable taste. Thus, a goal of scientists working to produce better milk products lies in providing bacteria which have all the characteristics associated with production of a good flavour and which are able to resist infection by bacteriophages.

A bacterium traditionally used in the production of milk products is S. thermophilus. It is particularly employed in the production of yogurt, mozzarella and Swiss type cheeses. One problem with S. thermophilus is that it is extremely sensitive to phage infection.

Little is known about natural phage resistance mechanisms of S. thermophilus. The bacterium was identified comparatively recently and, to date, very little data relating to the bacterium has been reported. In addition, it seems that S. thermophilus has fewer antiphage mechanisms than other bacteria eg L. lactis. In *Lactococcus lactis*, the major mesophilic starter of the dairy industry, natural phage-resistance mechanisms are abundant and usually encoded on conjugative plasmids. These have been used to construct food-grade phage resistance mechanisms into important industrial lactococcal starters. Information now obtained from phage genome analysis has shown no pausity in restriction sites as expected when phages are exposed to restriction/ modification selection. Moreover, in contrast to L. lactis, where the majority of antiphage mechanisms have been linked to plasmids, S. thermophilus has a comparatively small number of plasmids.

Therefore, a need exists for a bacterium which has the characteristics associated with production of a good flavour and which is able to resist infection by bacteriophages. In addition, there is a need for a method of providing bacteria with resistance to phage attack thereby enabling strains to be selected on the basis of their ability to produce a good flavour rather than their ability to resist bacteriophages.

Spontaneous phage-insensitive mutants (PIM) can be selected by phage challenge and this approach has been applied to *S. thermophilus*. However, such mutants are usually slow acid producers and can also revert to phage sensitivity.

The present invention addresses the problems set out above.

Remarkably, molecular methods have now been exploited to create phage-resistant strains. A new and suprising approach based on inactivation of a bacterial host gene has proven successful in inactivation of prophages by bacterial gene inactivation by targeting them with food-grade plasmid(s). A second new and suprising approach is based on the protection provided by at least partial deletion of the Sfi21 prophage. Temperate phages usually code for superinfection immunity genes, which are quite effective in protecting the lysogen from superinfection by both temperate and lytic phages. In the case of phage Sfi21 the superinfection control appears to be mediated by two distinct genetic elements: orf 203, a superinfection immunity gene and orf 127, the phage repressor. A drawback is that Sfi21 lysogens continuously release infectious phage particles into the media, which could contaminate the factory and possibly prevent a later introduction of valuable starters susceptible to phage Sfi21. It has also been shown that temperate phages can be the source of lytic derivatives. Therefore, targeted deletions in the Sfi21 prophage were created and inserted in the known lysogenic starter Sfi1cl6 to obtain a lysogen that would retain superinfection control, but has lost its capacity to produce infectious virions. Both approaches can provide powerful phage-resistant food-grade starters for industrial milk fermentation.

Consequently, in a first aspect the present invention provides a bacterium which is resistant to attack by at least one bacteriophage that comprises a modification of the Sfi21 prophage or the bacterial chromosome.

In a second aspect the invention provides a method of preparing a bacterium according to the invention which comprises the steps of disrupting the Sfi21 prophage or the bacterial chromosome by inserting or deleting a sufficiently large DNA sequence from the gene.

In a third aspect the invention provides a composition which comprises the bacterium according to the invention together with a carrier, adjuvant or diluent.

Preferably, an embodiment of the bacterium according to the present invention is a S. thermophilus bacterium. More preferably it is a strain of S. thermophilus selected from the group which consists of Sfi1 and Sfi1c16.

Preferably, an embodiment of the bacterium according to the invention comprises a modified bacterial chromosome. More preferably, the bacterial chromosome comprises additional DNA. Preferably, the additional DNA comprises the sequence of bases of ISS1 (see J. Bacteriol 178, 931-935 (1996)) or a functional equivalent thereof. More preferably the additional DNA is introduced to the bacterial chromosome in ORF 90 at a site which disrupts expression of a chorismate mutase chain A gene and/or disrupts expression of the down stream gene ORF 394; or in ORF 269 at a site which disrupts expression of an oxidoreductase gene.

Preferably, an alternative embodiment of the bacterium according to the present invention has a modified Sfi21 prophage which is modified by addition or deletion of sufficient DNA to disrupt expression of the prophage. More preferably the modification is a deletion. Even more preferably the modification comprises a deletion of at least part of ORF 1560.

Preferably, an embodiment of the method according to the invention comprises the step of modifying the bacterial chromosome to disrupt the expression of one or more genes which are required by a bacteriophage, but not essential for the bacterium. More preferably, the method includes the step of adding DNA to the chromosome. Preferably, the method comprises the step of adding DNA which comprises the sequence of bases of ISS1 (see J. Bacteriol 178, 931-935 (1996)) or a functional equivalent thereof. More preferably the method comprises the step of adding DNA to the bacterial chromosome in ORF 90 at a site which disrupts expression of a chorismate mutase chain A gene or in ORF 269 at a site which disrupts expression of an oxidoreductase gene.

Preferably, an alternative embodiment of the method according to the present invention comprises the step of modifying the Sfi21 prophage. Preferably it comprises the step of adding or deleting a sufficient amount of DNA to disrupt expression of the prophage. More preferably the modification is a deletion. Even more preferably the modification comprises the step of deleting at least part of ORF 1560.

Preferably, an embodiment of the composition according to the present invention comprises an embodiment of the bacterium together with a carrier, adjuvant or diluent. More preferably it is a starter culture or milk product.

An advantage of the present invention is that it provides a bacterium which is resistant to attack by bacteriophages.

Another advantage of the present invention is that it provides a bacterium which does not revert to bacteriophage sensitivity and the method by which it is produced is generally applicable.

Yet another advantage of the present invention is that it provides bacteria having such a high level of phage resistance that the obtained phage-resistant bacteria do not allow spontaneous development of mutated phages that could infect the bacteria. This is not the case for other phage resistance mechanisms. Thus, these bacteria should be protected over a long period before new phages develop, which could infect them.

Yet another advantage is that bacteria have been obtained which have resistance to attack by a broad range of phages. The wild type Sfi1 bacterial strain is extremely susceptible to phage attack. It can be infected by more than 25 different phages (normal strains are susceptible to 1 or 2 phages). In stark contrast, no phages were able to infect the mutated Sfi1 strains according to embodiments of the invention. It is important to stress that the stability of the bacteriophage resistance phenotype of chromosomally modified bacteria is in contrast to the weak resistance of bacteria that have plasmid encoded phage resistance mechanisms. Moreover, there are presently no known food-grade plasmids available that may be used to provide phage resistance in *S. thermophilus*.

Additional features and advantages of the present invention are described in, and will be apparent from, the detailed description of the presently preferred embodiments which are set out below with reference to the drawings in which:

Figure 1 shows results of a Southern blot of total DNA of phage resistant integrands. Lanes 1-5: phage resistant Sfi1::pG+host9IS*S1*. Lane 6: Sfi1. M:

DNA marker (λ-DNA x HindIII). The blot was probed with radioactively marked pG+host9IS*S1* plasmid and λ-DNA. Note: this figure is the combination of two independent blots (lane 1 has been added).

Figure 2 shows the results of detection of intracellular phage DNA. The numbers correspond to the time in minutes after which samples have been taken (see Materials and methods). The two lines marked with M correspond to the size marker (1 kb DNA Ladder, GibcoBRL).

Figure 3 shows growth curves of phage-resistant *S. thermophilus* strains compared to the parental strain. 1: Sfi1. 2: Sfi1-R7. 3: Sfi1-R71. 4: Sfi1-R24.

Figure 4 shows sites of pG$^+$host9IS*S1* insertion in Sfi1-R7 and Sfi1-R24. Open arrows indicate the predicted open reading frames (orf). The numbers indicate the number of codons for each orf. When possible, a tentative function has been attributed to the predicted gene products.

Figure 5 shows deletion in Sfi1-R7e. The sequence deleted in Sfi1-R7e is in bold. The arrow indicates the pG$^+$host9IS*S1* integration site.

Figure 6 shows growth curve of *S. thermophilus* Sfi1cl6D1560 (2) compared to Sfi1cl6 (1).

Figure 7 shows a genomic map of the phage Sfi21. It shows annotation of a gene corresponding to ORF 1560 which may be deleted, at least partially. It codes for a putative tail length determining protein which is required for phage mutagenesis.

Without wishing to be bound by theory it is postulated that the presence of some bacterial genes are essential to phage development, but dispensable for bacterial growth in milk. In fact, it is now thought that bacteriophages depend on host factors in many steps of their life cycle, e.g. adsorption, DNA injection, replication and morphogenesis. Disruption of one of these factors is now considered to lead to a phage-resistant cell.

Having no knowledge of the *S. thermophilus* genome, random gene inactivation was carried out with the temperature sensitive plasmid pG$^+$host9IS*S1*. The method is self-selective: after phage challenge non-resistant cells are eliminated and mutants leading to decreased bacterial fitness are outnumbered.

Materials and methods

Strains, media, plasmids, and culture conditions were as follows. The *E. coli* strain 101 was propagated in LB broth or LB broth solidified with 1.5% (W/V) agar at 37°C. Liquid cultures were grown under agitation (240 rpm). *S. thermophilus* strains Sfi1, its lysogenic derivative Sfi1cl6 (containing the Sfi21 prophage) and their transformants were cultivated at 42°C either in M17 supplemented with 0.5% lactose (LM17), Belliker media or MSK. Erythromycin was used when required at a final concentration of 2 and 150 µg/ml for *S. thermophilus* and *E.coli*, respectively. The phages used in this study were obtained from the Nestlé Culture Collection and propagated on their appropriate *S. thermophilus* strain in LM17 broth as described previously. Phage enumeration was achieved as described previously. For random insertion mutagenesis and directed mutagenesis, plasmids pG^+host9IS*S1* and pG^+host9 have been used, respectively.

DNA techniques

Phage purification, DNA extraction and purification, agarose gel electrophoresis, Southern blot hybridization, and DNA labelling were executed as described previously. The Qiaprep plasmid kit (Qiagen) was used for the rapid isolation of plasmid DNA from *E. coli*. Restriction enzymes and T4 ligase were purchased from Boehringer-Mannheim and used according to the supplier's instructions. *E. coli* was electrotransformated as outlined in the BioRad instruction manual. *S. thermophilus* was electroporated using the procedure described by Slos et al. The analysis of intracellular phage DNA, PCR and DNA sequencing have been performed as described previously.

Sequence analysis

The Genetics Computer Group (University of Wisconsin) sequence analysis package was used to assemble and analyze the sequences. Nucleotide and predicted amino acid sequences were compared to those in commercially available databases (GenBank, release 109, EMBL, release 56; PIR-Protein, release 57; SWISS-PROT, release 36; and PROSITE, release 15.0) with FastA and BLAST programs(2). Prediction of transmembrane domains was performed using the TMpred program.

Construction of plasmid for site directed integration

To create a deletion in the Sfi21 prophage, the thermosensitive plasmid pG$^+$host9AB1560, a derivative of pG$^+$host9 has been created. Two fragments of approximately 500 bp, A and B, were chosen in the orf1560 on the prophage sequence at a distance of 2.4 kb so that a deletion of the same size would be created by homologous recombination. Fragment A was generated by PCR using phage Sfi21 DNA as a template and primers (5'-AAC TGC AGT CTC AGC TCA AAG GGA C-3' and 5'-GGA ATT CTA GCC GTG ATG TTT TTG-3') containing *Pst*I and *Eco*RI restriction sites (underlined). Fragment B was generated by PCR using primers (5'-GGA ATT CGA CGC AAT TAA AGA CCC-3' and 5'-CCA TCG ATC TGC TTC CAA AAT CTC G-3') containing *Eco*RI and *Cla*I restriction sites. Both clones were then cloned into pG$^+$host9, so to be adjacent, generating the construct pG$^+$host9AB1560. The construct was first generated into *E. coli* 101, then transformed into *S. thermophilus* Sfi1cl6.

Transposition of pG$^+$host9IS*S1* and pG$^+$host9AB1560 in the *S. thermophilus* chromosome

S. thermophilus Sfi1 and Sfi1cl6 containing pG$^+$host9IS*S1* and pG$^+$host9AB1560, respectively, were grown overnight in LM17 medium supplemented with 2 µg/ml of erythromycin. The saturated cultures were diluted 100-fold in LM17 medium containing 1 µg/ml of erythromycin and incubated 2 h at 30°C. The cultures were then shifted to 42°C to eliminate free plasmids and grown to saturation. The frequency of integration per cell was estimated as the ratio of the number of Em^R cells at 42°C to the number of viable cells at 30°C. Integration of the plasmids was checked by Southern blot hybridization. To excise the transposed vectors, serial passages have been performed in LM17 broth without antibiotic.

Selection of phage-resistant mutants

The culture containing the original population of Sfi1::pG$^+$host9IS*S1* integrants was diluted 100-fold in LM17 supplemented with 2 µg/ml of erythromycin and challenged with lytic phage Sfi19 at a M.O.I. of 5. The culture was then grown to

saturation. The experiments were considered unsuccessful when no growth was observed after 48h.

Individual phage-resistant colonies were isolated; their total DNA was extracted, and digested with EcoRI, and Southern blots were performed using the plasmid DNA as probe. Three different hybridization patterns were identified, corresponding to the Sfi1-R7 (lane 3), Sfi1-R24 (lane 1) and Sfi1-R71 (lanes 2,4,5) insertion mutants (Fig. 1).

Phage adsorption test

S. thermophilus cultures were grown in LM17 until an OD_{600} of 0.6 was reached. Then phages were added at a M.O.I. of 1 and the cultures incubated at room temperature. Probes were taken immediately after phage addition and after 30 min. These probes were then filtered to remove the bacterial cells from the cultures. Phages left were then enumerated. The adsorption was calculated as phage counts at time 30 divided by the phage counts at time zero.

Results

Random mutagenesis of *S. thermophilus* Sfi1 was carried out. Starter strain Sfi1 was the best indicator cell. It is susceptible to about 25 of the 100 phages from the Nestle Culture Collection. This allows testing mutant starters against a broad range of phages.

Transposition of the plasmid could be achieved with a very high integration frequency, about 50% (see materials and methods). Integrants were randomly chosen on agar-plates and grown in liquid cultures. Their total DNA was then extracted to check by Southern blot hybridization the integration of the plasmid into the chromosome. Hybridization was performed on *Eco*RI digested chromosomal DNA using labelled pG$^+$host9IS*S1* as probe. All integrants showed signals corresponding to distinct chromosomal fragments (data not shown), confirming the integration of the plasmid and the randomness of the transposition.

Selection of phage-resistant mutants

Integrants were challenged with the lytic phage Sfi19 (M.O.I.=5) to select for phage-resistant mutants. In presence of phage Sfi19 the Sfi1::pG$^+$host9IS*S1* culture showed a delayed growth, an OD_{600} of 0.85 was only reached after 8 to 12 h of incubation in comparison with 3 h for the transformants in the absence of challenge phage. In contrast, no growth was observed for the parental Sfi1 starter after phage challenge (OD_{600} < 0.02 after 48 h). In fact, in LM17 medium we consistently failed to obtain phage resistant mutants of starter Sfi1 after challenge with numerous phages.

Three mutants were selected and designated Sfi1-R7, Sfi1-R24 and Sfi1-R71.

Characterization of the phage-resistant mutants

The 3 mutants were tested for phage resistance against 15 different *S. thermophilus* phages. In no case, phage plaques were observed suggesting in some cases efficiency of plating of < 10^{-7} (Table 1).

Phage	Sfi1 (pfu/ml)	Sfi1-R7 (pfu/ml)	Sfi1-R24 (pfu/ml)	Sfi1-R71 (pfu/ml)
Sfi21	6 X 10^8	<10^2	<10^2	<10^2
S69	7.2 X 10^3	<10^2	<10^2	<10^2
Sfi3J	2 X 10^8	<10^2	<10^2	<10^2
St44A	1 X 10^8	<10^2	<10^2	<10^2
S19	2 X 10^8	<10^2	<10^2	<10^2
S96	4 X 10^7	<10^2	<10^2	<10^2
St44	1 X 10^8	<10^2	<10^2	<10^2
St40	2 X 10^5	<10^2	<10^2	<10^2
Sfi18	2.7 X 10^7	<10^2	<10^2	<10^2
Sfi19	1.1 X 10^9	<10^2	<10^2	<10^2
St25	3 X 10^8	<10^2	<10^2	<10^2
St17	1 X 10^8	<10^2	<10^2	<10^2
S17	6 X 10^8	<10^2	<10^2	<10^2
H	4.5 X 10^3	<10^2	<10^2	<10^2
F	1 X 10^4	<10^2	<10^2	<10^2

Table 1

To get an indication of the stage at which phage development is blocked, phage-adsorption and phage DNA-replication tests were performed. The adsorption test was performed using Sfi19 on Sfi1 and the three mutants. After 30', 90% of the input phages adsorbed to Sfi1, 94% to Sfi1-R7, 91% to Sfi1-R24 and 87% to Sfi1-R71. This indicates that the mutation did not affect phage adsorption.

DNA replication of phage Sfi19 in Sfi1 and the mutants was analyzed by determining the relative amount of intracellular phage DNA at different times during phage infection. In Sfi1 phage DNA was readily detected 20 min after infection (Fig. 2). The amount of DNA increased to a maximum after 40 min. Then a decrease was observed after 60 and 80 min, probably because of phage induced cell lysis. Phage DNA replication in the Sfi1-R24 mutant was delayed and decreased.

Phage DNA was detected only after 40 min reaching a maximum at 60 min. In the case of Sfi1-R7 and Sfi1-R71 no phage replication was observed. A very low and invariable level of phage DNA was detected by hybridization. This could represent the injected DNA of the infecting input phages or DNA from uninjected, but adsorbed input phages. The growth of the mutants was compared to that of the wild type strain in milk. This was done by measuring changes in impedance of the culture media (Rapid Automated Bacterial Impedance Technique, Don Whitley Systems). This system measures indirectly the transformation of a weak electrical conductor like the polar, but uncharged lactose into the electrically charged lactic acid during bacterial growth. The resulting curve (time vs. conductivity) can be correlated with both bacterial growth and acidification of the culture. All three mutants did not show significant differences in growth (and acidification) to the parental strain Sfi1 (Fig. 3).

Determination of the site of integration

The fragments adjacent to the insertion point were obtained by plasmid rescue as described by Maguin et al. for the Sfi1-R7 and Sfi1-R24 mutants. The approach was unsuccessful for Sfi1-R71. The regions flanking the pG$^+$host9IS*S1* insertion point were sequenced for the two mutants (Fig. 4).

In Sfi1-R7 the plasmid integrated in the last 4 amino acids of a putative chorismate mutase chain A gene (PheA), orf 90. This site of integration could also function as promoter region for the downstream orf 394. Orf 394 gene product shows similarities (26% identity) to a hypothetical conserved protein of *Methanococcus jannashii* (Accession number MJ0305). Orf 394 gp may be an integral protein since 9 strong transmembrane helices were predicted. Orf 115 gp encoded by a gene located directly downstream of orf 394, showed significant

similarity to the ribosomal protein L19 from a number of bacteria (*Haemophilus influenzae*, *Salmonella typhimurium*, *Serratia marcescens*, *Synechocystis sp.*). A gene almost identical to a tRNAArg gene (anticodon CCU) follows this gene from *E. coli* and interestingly the phage attachment site *att*B for prophage integration. Upstream of orf 90 a gene we identified orf 486 (see Fig. 4), which encodes a further possible membrane protein. The deduced protein for this orf shows significant similarity (50% over 427 aa) to a hypothetical Cl-channel-like protein from *E. coli* (Accession number P37019).

In Sfi1-R24 the pG$^+$host9IS*S1* insertion point was in a likely oxidoreductase involved in fatty acid biosynthesis (orf 269). The predicted protein showed 42% amino acid identity with an *E. coli* 3-oxoacyl-[acyl-carrier protein] reductase.

Interestingly, one fragment of an R-subunit of a type I restriction enzyme is located downstream orf 269. No database matches were found for the orfs upstream of orf 269.

Plasmid excision.

To obtain food-grade starter strains it is necessary to remove the erythromycin resistance gene present in the transformants. IS*S1* undergoes replicative transposition which leads to an integrated plasmid vector flanked by two IS elements. Therefore, homologous recombination between this two copies of the IS element will entirely remove the plasmid and leave only a single copy of the IS element. To favor recombination, serial passages of the transformants were done in absence of antibiotic selection. Sfi1-R24 lost the Em^R phenotype after 29 passages (now Sfi1-R24e), Sfi1-R71 after 40 (Sfi1-R71e). Excision was easier with Sfi1-R24, because of the lack of tandem repeats (Fig. 1). Sfi1-R7 Em^S (Sfi1-R7e) could be obtained after 60 passages. Then, phage resistance was checked for the Em^S mutants. In 2 cases (Sfi1-R24 and Sfi1-R71) the loss of the plasmid resulted in full reversion to phage sensitivity while Sfi1-R7e did retain its full phage resistance phenotype. This derivative lacked the plasmid sequence except for one IS*S1* sequence. Sequencing of the flanking sequences of the remaining IS*S1* showed in addition a 69 bp deletion which started at the IS transposition site and removed 3'-end of orf 90 and the first 15 codons of orf 394 (Fig. 5). Since two independently obtained Sfi1-R7e mutants showed the same deletion it is likely that the deletion has already occurred in the parental Sfi1-R7 mutant.

Deletion of orf 1560 from the Sfi21 prophage

Integration of pG$^+$host9AB1560 into the prophage was readily obtained (see materials and methods). Integration in the right site was checked by PCR. More than 50% of the integrands were shown to give a signal corresponding to the

predicted size for homologous recombination events. Plasmid excision was obtained after 20 serial passages. Because the second homologous recombination can lead both to the wild type or the deletion mutant, PCR was used to test the Em^S clones. As expected about 50% of the clones were deletion derivatives (Sfi1cl6Δ1560).

Characterization of Sfi1cl6Δ1560

To test if an essential gene of the phage had been inactivated the prophage of both Sficl6 and Sfi1cl6Δ1560 was induced with 2 µg/ml of Mytomicin C. Sficl6 released 10^5 pfu/ml, Sfi1cl6Δ1560 released no detectable infectious particles. Subsequently, it was checked whether the superinfection exclusion phenotype was preserved in the derivative lysogen.

Phage	Sfi1 (pfu/ml)	Sfi1cl6 (pfu/ml)	Sfi1cl6Δ1560 (pfu/ml)
Sfi18	7.4×10^7	$< 10^2$	$< 10^2$
S96	9.8×10^6	$< 10^2$	$< 10^2$
Sfi21	8.3×10^7	$< 10^2$	$< 10^2$
Sfi19	1.6×10^8	8.8×10^5	1.7×10^6
ST25	1.1×10^8	1.6×10^7	2.2×10^7
ST17	6.5×10^8	1.2×10^4	1.3×10^4

Table 2

In fact, plaque assays demonstrated an identical phage exclusion phenotype in both with respect to suppression of phage infectivity expressed as efficiency of plating as well as in the range of different phages which were inhibited (Table 2). Growth curves of the lysogen and its deletion derivative were identical (Fig. 6).

The invention provides an increase in phage resistance of *S. thermophilus* starter strains. Random insertion of an integrative plasmid into the chromosome of *S. thermophilus* led to production of highly phage resistant (e.o.p. $< 10^{-6}$) mutants.

The broad range of anti-phage activity of the Sfi1-R7 mutation suggests that the 15 phages, which were tested, have similar requirements for host factors; i.e. orf 90 and orf 394 gps. At the level of analysis achieved it is not possible to exclude effects of the IS element and/or the adjacent deletion on the transcription of adjacent bacterial genes. Phage structural proteins interact with bacterial structures at least for phage adsorption and DNA injection. The apparent lack of intracellular phage DNA in the Sfi1-R7 and Sfi1-R71 mutants may be an indication of interference with phage DNA injection. In phages from Gram-positive bacteria initial phage adsorption and phage DNA injection seem to follow a two step process. First, phages adsorb reversibly to carbohydrate

components of the cell wall, then appears to follow an irreversible interaction with the plasma membrane and ejection of DNA. For bacteriophage c2 it has been shown that a membrane protein, PIP, was responsible for irreversible phage adsorption to the host. Phage c2 was unable to infect cells with defective PIP proteins. Whether PIP is also responsible for phage DNA injection or other structures are needed is not known. For example, bacteriophage λ requires a host protein (Pel) in addition to the LamB receptor for an effective DNA injection. Pel is apparently not involved in phage adsorption since λ binds tightly to *E. coli pel*⁻ strains. It is thus possible that in the case of the Sfi1-R7 and Sfi1-R71 mutants, proteins involved in phage irreversible adsorption and/or DNA injection have been modified. In fact, the sequence information available for Sfi1-R7 indicates that the insertion of pG⁺host9IS*S1* may have blocked the expression of a putative integral membrane protein.

The phage resistance phenotype of Sfi1-R24 resembles that of an abortive infection mechanism. The phage DNA can enter the cell and phage DNA replication takes place, but it is delayed and diminished. In view of this it is considered that the insertion event affects the expression of a host factor involved in a phage multiplication step preceding DNA replication. Blockage of this step could affect DNA replication negatively. In the case of Sfi1-R24, the oxidoreductase is not the host factor whose inactivation causes the delay in DNA replication since its interruption by the IS element after plasmid excision fails to provide phage inhibition. Apparently, the transcription of adjacent genes is disturbed by the integrated plasmid. A gene encoding a subunit of a restriction endonuclease was the only bioinformatic hit in the targeted region. However, restriction and modification mechanisms are excluded since no traces of phage DNA degradation are detected in the Southern blot (Fig. 2). Direct interference with phage DNA replication is unlikely since the inhibited phages have apparently different DNA replication modules and are thus likely to depend on different host replication functions.

The Sfi21 prophage could be inactivated by targeted food-grade phage gene inactivation. No release of infectious particles by the Sfi1cl6Δ1560 lysogen could be detected after prophage induction. Importantly, the inactivated prophage retained its protective effect against superinfection with a broad range of temperate and lytic *S. thermophilus* phages. Consequently, inactivated lysogens can now be considered as valuable starters. Their protective power exceeds that obtained with starter strains containing phage-inhibitory plasmids. For example, the superinfection immunity gene of Sfi21 (orf 203) was cloned in a high copy number plasmid and tested for protection against phages. It was quantitatively and qualitatively less complete than that mediated by the prophage. In addition, the plasmid was no longer phage inhibitory when integrated as a single copy into the bacterial chromosome. Therefore, the use of orf 203 has to rely on its

presence on a high copy number plasmid, with the problem of high metabolic costs and plasmid instability. In contrast, the integrated prophage seems to be of low metabolic cost to the cell since lysogenic and non-lysogenic *S. thermophilus* starters have identical growth properties. Inactivated prophages can be used to increase the phage resistance of other strains than Sfi1 since a number of valuable industrial *S. thermophilus* starters can be lysogenized with phage Sfi21.

It should be understood that various changes and modifications to the presently preferred embodiments described herein will be apparent to those skilled in the art. Such changes and modifications can be made without departing from the spirit and scope of the present invention and without diminishing its attendant advantages. It is therefore intended that such changes and modifications be covered by the appended claims.

Claims

1. An S thermophilus bacterium which is resistant to attack by at least one bacteriophage and which comprises a modification of the Sfi21 prophage or the bacterial chromosome.

2. A bacterium according to claim 1 which is a strain of S. thermophilus selected from the group which consists of Sfi1 and Sfi1c16.

3. A bacterium according to claim 1 or claim 2 wherein the bacterial chromosome is modified by addition of DNA.

4. A bacterium according to claim 3 wherein the addition of DNA comprises the sequence of bases of ISS1 or a functional equivalent thereof.

5. A bacterium according to claim 3 or 4 wherein the bacterial chromosome comprises addition of DNA in ORF 90 at a site which disrupts expression of a chorismate mutase chain A gene and/or disrupts expression of the down stream gene ORF 394; or in ORF 269 at a site which disrupts expression of an oxidoreductase gene.

6. A bacterium according to claim 1 or 2 wherein the Sfi21 prophage is modified by deletion of sufficient DNA to disrupt expression of the prophage.

7. A bacterium according to claim 6 wherein the modification comprises a deletion of at least part of ORF 1560.

8. A method of preparing a bacterium according to any one of claims 1 to 7 which comprises the steps of disrupting expression of the bacterial chromosome or the Sfi21 prophage by inserting a DNA sequence or deleting a DNA sequence.

9. A composition which comprises the bacterium according to any one of claims 1 to 6 together with a carrier, adjuvant or diluent.

10. A composition according to claim 8 which is a starter culture or milk product.

Figure 1

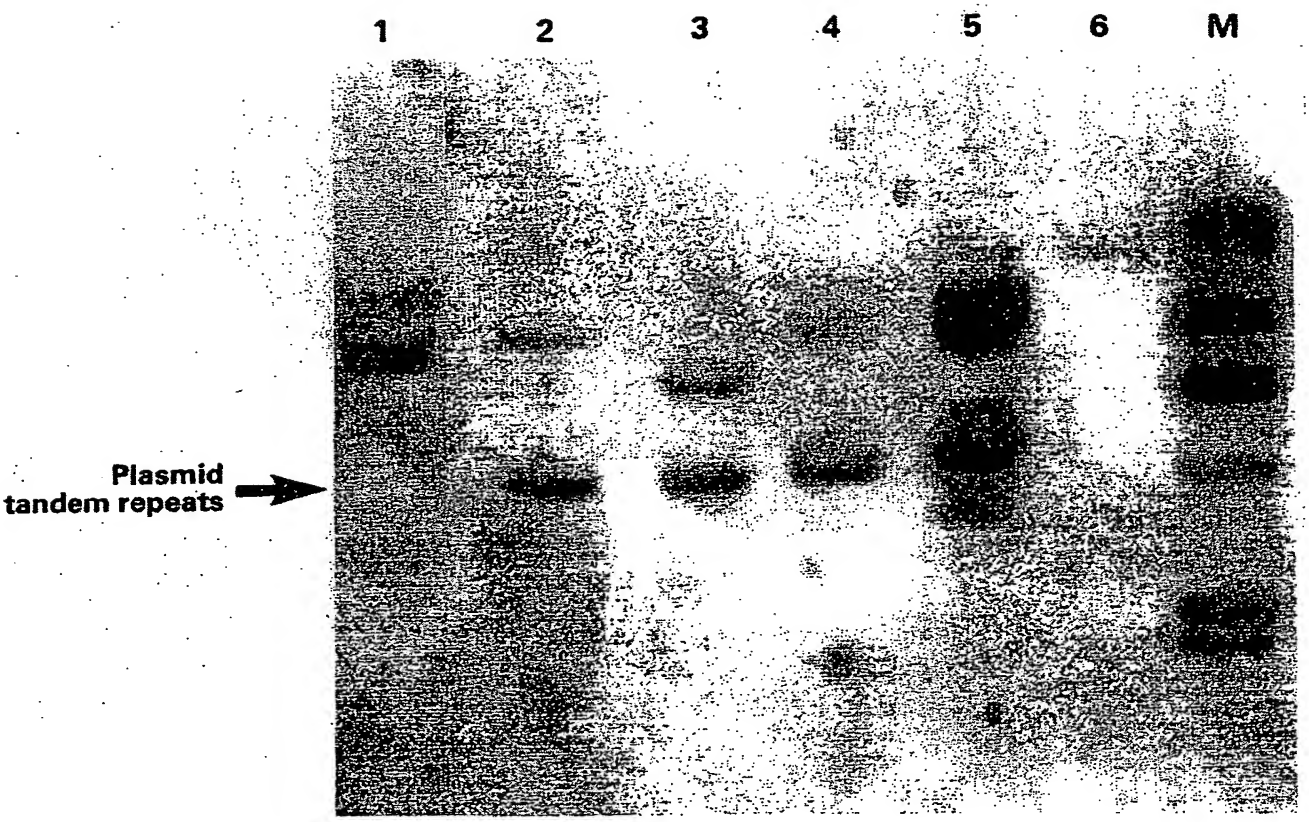
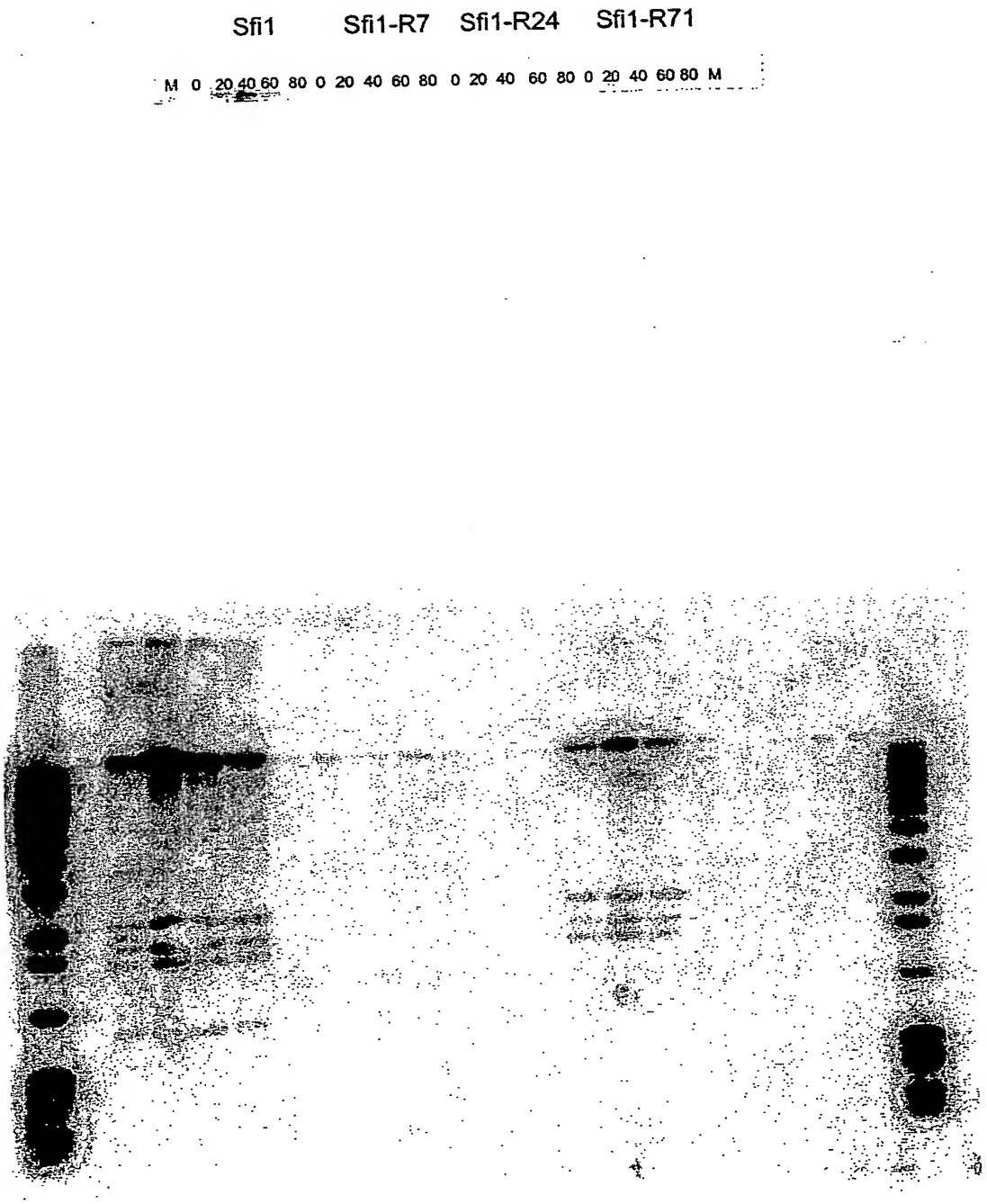


Figure 2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Sfi1-R24
Sfi1-R71
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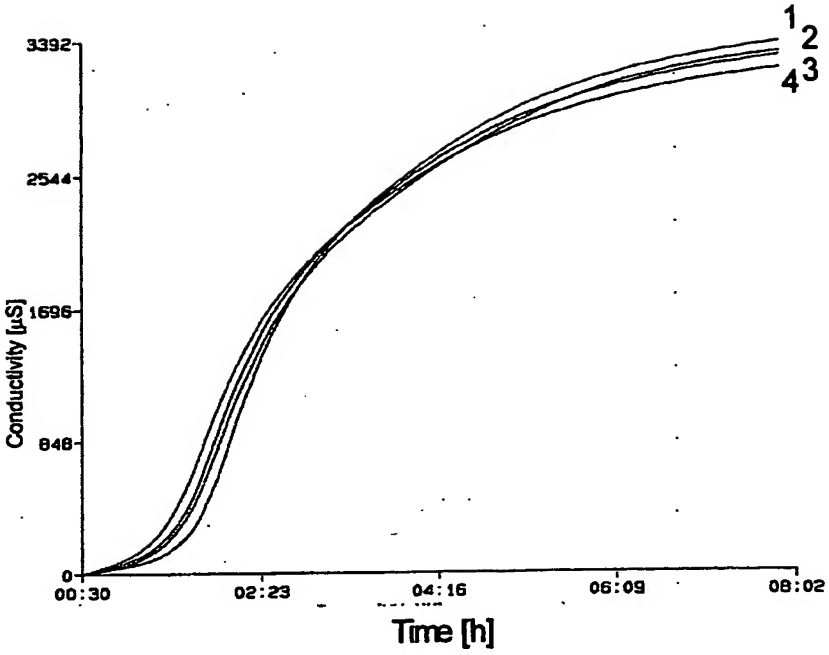
Figure 3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1696
848
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Conductivity [µS]
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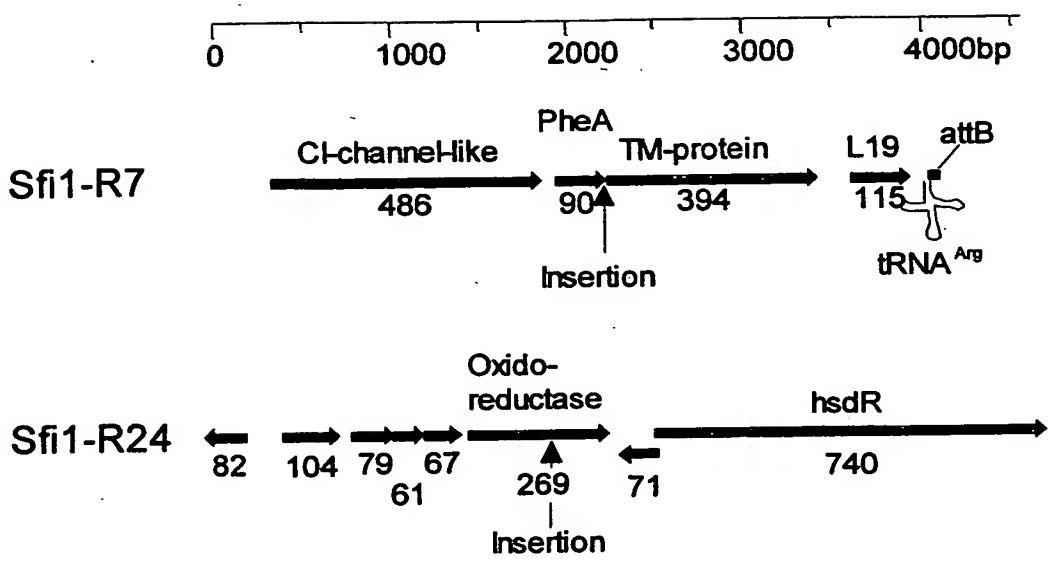
02:23
04:16
06:09
08:02
Time [h]
1
2
3
4

Figure 4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TM-protein
L19
attB
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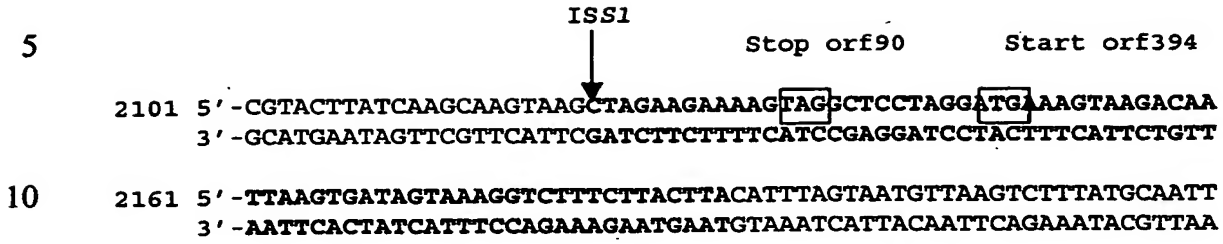
740
Insertion

Figure 5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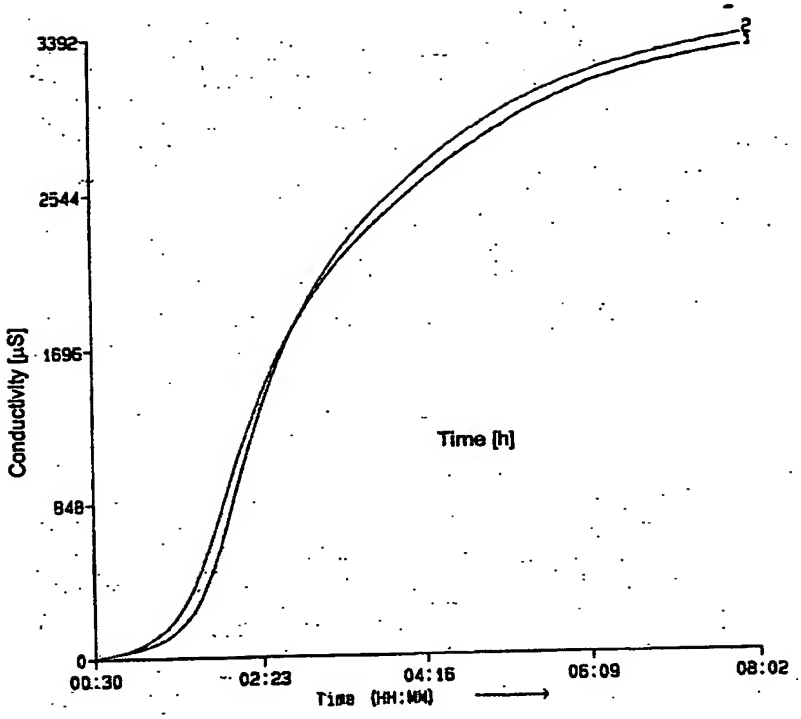
3'-AATTCACTATCATTTCCAGAAAGAATGAATGTAAATCATTACAATTCAGAAATACGTTAA

Figure 6


3392
2544
1696
848
0
Conductivity [µS]
Time [h]
00:30
02:23
04:16
06:09
08:02
Time (HH:MM)
2
1

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(71) Applicant *(for all designated States except US)*: SOCIETE DES PRODUITS NESTLE S.A. [CH/CH]; P.O. Box 353, CH-1800 Vevey (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BRUESSOW, Harald [DE/CH]; 13, Chemin de la Chaumény, CH-1814 La-Tour-de-Peilz (CH). LUCCHINI, Sacha [CH/GB]; 43, The Close, Norwich NR1 4EQ (GB).

(74) Agent: LOCK, Graham; 55, Avenue Nestlé, CH-1800 Vevey (CH).

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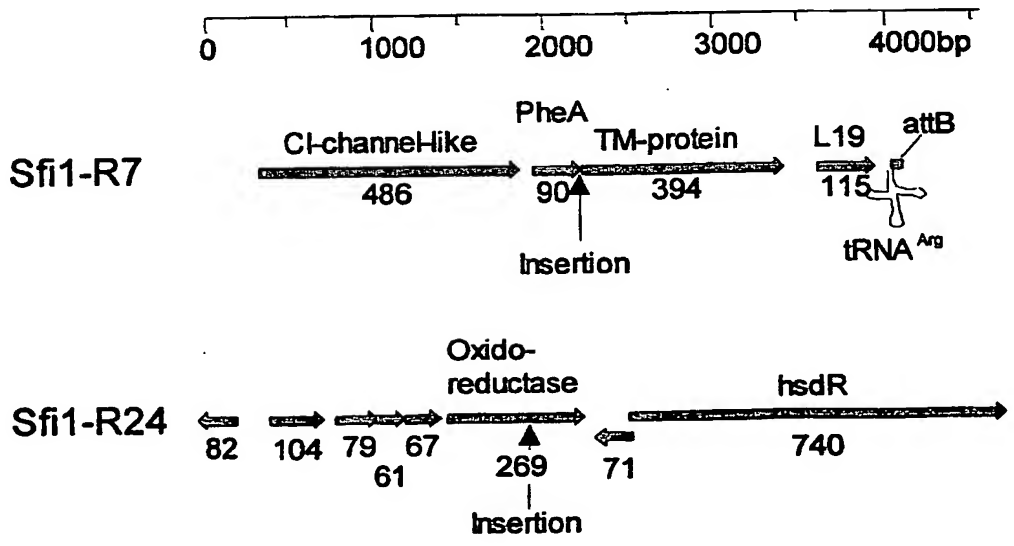
(54) Title: PHAGE RESISTANT STREPTOCOCCUS THERMOPHILUS



(57) Abstract: The invention relates to an S thermophilus bacterium which is restant to attack by at least one bacteriophage, a method of production of the bacterium and a composition which comprises the bacterium. The bacterium comprises a modification of the Sfi21 prophage or the bacterial genome which disrupts expression of a gene which is essential to a bacteriophage, but not essential to the bacteria.

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INTERNATIONAL SEARCH REPORT

Int. [redacted] onal Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/31 C12N1/21 A23C9/12 A23C19/032 //(C12N1/21, C12R1:46)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A23C

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, FSTA, MEDLINE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STURINO J M ET AL: "Construction of bacteriphage resistant strains of Streptococcus thermophilus by pGh9::ISS1 insertional mutagenesis." JOURNAL OF DAIRY SCIENCE, vol. 81, no. SUPPL. 1, 1998, page 7 XP002163735 Joint Meeting of the American Dairy Science Association and the American Society of Animal Science;Denver, Colorado, USA; July 28-31, 1998 ISSN: 0022-0302 the whole document	1,3,4, 8-10
X	EP 0 748 871 A (NESTLE SA) 18 December 1996 (1996-12-18) claims 1-9; examples 1-4	1-3,6, 8-10

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[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Devijver, K

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INTERNATIONAL SEARCH REPORT

Ir. [redacted]onal Application No
PCT/EP 00/07696

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	FOLEY SOPHEY ET AL: "A short noncoding viral DNA element showing characteristics of a replication origin confers bacteriophage resistance to Streptococcus thermophilus." VIROLOGY, vol. 250, no. 2, 25 October 1998 (1998-10-25), pages 377-387, XP002163736 ISSN: 0042-6822 page 379 -page 380; figure 1; tables 1-3 ---	1-3,6,8
A	EP 0 183 469 A (GENENTECH INC) 4 June 1986 (1986-06-04) ---	
A	MAGUIN E ET AL: "EFFICIENT INSERTIONAL MUTAGENESIS IN LACTOCOCCI AND OTHER GRAM-POSITIVE BACTERIA" JOURNAL OF BACTERIOLOGY,US,WASHINGTON, DC, vol. 178, no. 3, 1 February 1996 (1996-02-01), pages 931-935, XP000673892 ISSN: 0021-9193 cited in the application ---	
T	LUCCHINI SACHA ET AL: "Broad-range bacteriophage resistance in Streptococcus thermophilus by insertional mutagenesis." VIROLOGY, vol. 275, no. 2, 30 September 2000 (2000-09-30), pages 267-277, XP002163737 ISSN: 0042-6822 -----	

INTERNATIONAL SEARCH REPORT

Information on patent family members

In [redacted]nal Application No
PCT/EP 00/07696

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0748871 A	18-12-1996	CA 2178975 A	17-12-1996
		JP 9000274 A	07-01-1997
		US 5766904 A	16-06-1998
EP 0183469 A	04-06-1986	JP 61132179 A	19-06-1986